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Isolation of a cDNA clone and characterization of expression of the highly abundant, cold acclimation-associated 14 kDa dehydrin of blueberry

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Abstract

Dehydrins are a family of plant proteins that are induced by dehydrative stresses such as those caused by drought, salinity, and low/freezing temperature. Induction of some dehydrins may be responsive to ABA and/or short photoperiod as well. Previously, we reported that in blueberry a family of dehydrins of 65, 60, and 14 kDa accumulates in floral buds during the winter, and the levels of these proteins correlate with cold tolerance. Decline in level of the 14 kDa dehydrin with exposure to warm temperatures correlates well with loss of hardiness or deacclimation also. In the present study, we identified and sequenced a cDNA clone from blueberry floral bud RNA that encodes the 14 kDa dehydrin. The identity of the clone was confirmed by comparing partial peptide sequences from the protein with deduced protein sequence from the cDNA. The cDNA was found to be a full-length 653 bp clone, comprised of a 55 bp 5′ UTR, an ORF of 303 bp, a 213 bp 3′ UTR, and an 82 bp polyA tail. The cDNA has 2 K boxes, indicative of dehydrins, and no Y or S segments; thus it was classified as a K_2 type dehydrin. Expression of the 14 kDa dehydrin was studied at the transcript and protein levels in stem and leaf tissues under induced cold and drought stress in two genotypes, 'Bluecrop' and 'Premier', which differ in terms of their cold and drought tolerances. Expression of the protein was monitored using a polyclonal antibody raised against a synthetic peptide of the consensus K box of blueberry dehydrins, which was found to cross react with many blueberry dehydrins. The 14 kDa dehydrin, like other K_n type dehydrins, was strongly induced by cold stress and to a lesser extent by drought stress. A previously uncharacterized 16 kDa dehydrin showed similar induction on Western blots; however, it appeared to increase over time during the course of the experiments in stems of the southern variety 'Premier'. Like some other dehydrins and bark storage proteins, expression of the 16 kDa dehydrin may be responsive to short photoperiods. Patterns of expression of the 14 kDa dehydrin at the protein level were very consistent with patterns at the RNA level, and the 14 kDa dehydrin message was induced to higher levels in the more cold hardy and drought tolerant genotype, 'Bluecrop', than in 'Premier'.
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Keywords: Cold stress; Drought stress; Freezing tolerance; *Vaccinium*; Cold hardness; Cold tolerance

1. Introduction

Dehydrins are a distinct group of plant proteins belonging to the group II subclass of late embryogenesis-abundant (LEA) proteins that are believed to play a protective role during cellular dehydration [1–3]. They accumulate during dehydrative stresses caused by or associated with low/freezing temperature, drought, salinity, embryo desiccation, and ABA. In woody perennials, some dehydrins are also induced by short photoperiod followed by up-regulation by low temperature [4,5]. Dehydrins are characterized by a highly conserved 15-mer lysine rich sequence, called a K segment, which may be present in one or several copies [1]. The K segment can form an amphiphathic α helix structure [6,7] that may have a chaperone-like function in stabilizing partially denatured proteins or membranes [2]. Apart from the K segment that is present in all dehydrins, dehydrins may also posses one or more Y (DEYGNP) and/or S segments (serine cluster) in their sequence. Dehydrins can thus be

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categorized into classes based on number and position of these conserved motifs, for example Y_nSK_2, K_n, K_nS, SK_n, and Y_2K_n types [1]. Although the exact role of dehydrins in plant tissues is still not very well understood, it is evident that different classes of dehydrins respond to different kinds of dehydrative stresses. For instance, generally the Y_nSK_2 type responds to ABA and dehydration but not low temperature, K_n and Y_2K_n types respond to low temperature primarily, the SK_n type responds to low temperature, salinity, drought, and wounding, and the K_nS type responds to drought and low temperature (reviewed by Allagulova et al. [8]).

Dehydrins are hydrophilic, thermostable proteins enriched with glycine, in addition to lysine, and have a wide range of molecular masses from 9 to 200 kDa [1,9]. A number of studies, both time-course and comparative of different genotypes, have found a positive correlation between dehydrin accumulation and drought and cold stress tolerance ([1,10–19]; among others). From recent studies on kinetics of protein and transcript accumulation of several low molecular weight dehydrins in various plant species, it appears that cold tolerance often best correlates with accumulation of these low molecular weight dehydrins [17,19,20–22].

Our laboratory is studying the genetics of cold hardiness in a woody perennial, blueberry *Vaccinium* spp. We have found three dehydrins, 65, 60, and 14 kDa, to be strongly induced by cold in floral buds and their accumulation to be correlated with levels of bud cold hardiness [11]. A full-length cDNA clone encoding the 60 kDa dehydrin was isolated previously from a library constructed from RNA of cold acclimated floral buds [16]. Accumulation of this 60 kDa, K_5 type, dehydrin and the related 65 kDa dehydrin was studied in leaves, stems, and roots in response to cold and drought stress using an antibody raised against the 65 kDa dehydrin, which cross reacts with the 60 and 65 kDa dehydrins, but not the 14 kDa dehydrin. The 60 and 65 kDa dehydrins appear to be strongly induced by cold and slightly, or not at all in some cases, by drought [18]. In addition, levels of the major blueberry dehydrins of 65, 60, and 14 kDa progressively decrease in their abundance during deacclimation (loss of cold hardiness) of floral buds. In particular, down-regulation of the 14 kDa dehydrin most closely mirrors the loss in cold hardiness during deacclimation, suggesting its involvement in regulation of bud dehardening [23]. Most recently, we have begun using a genomic approach to better understand cold hardiness in blueberry by developing expressed sequence tags (ESTs) from two cDNA libraries constructed from RNA isolated from cold acclimated and non-acclimated floral bud tissues [24]. A comparison of the ESTs from the two libraries, followed by Northern analysis, has resulted in the identification of six new cold acclimation-responsive genes in addition to the previously characterized dehydrin genes. Several dehydrin cDNAs, most of which appear to encode low molecular weight dehydrins, were identified as well. Here, we report the identification and sequencing of a full-length cDNA clone encoding the 14 kDa dehydrin. Also, we describe the expression of the 14 kDa dehydrin at the protein and transcript levels, under cold and drought stress, in leaf and stem tissues of two genotypes which differ in terms of their cold and drought tolerances.

2. Materials and methods

2.1. Plant material

To study expression of dehydrins in blueberry floral buds throughout the fall and winter, buds were collected from field plants of the cultivar 'Bluecrop' (*V. corymbosum* L.) that had accumulated 0 (collected 10/2/02), 400 (12/4/02), 800 (1/8/03), and 1200 (2/26/03) chill units. Here, one chill unit equaled 1 h of exposure to temperatures between 0 and 7 °C. Field plants were maintained at the Henry A. Wallace Agricultural Research Center at Beltsville, MD.

To study expression of the 14 kDa dehydrin in leaves and stems in response to imposed cold and drought stress, 4-year-old potted blueberry plants of two genotypes, 'Bluecrop' and 'Premier' (*V. ashei* Reade), were maintained in a greenhouse under natural photoperiod without watering for the drought experiment, and in a cold room at 4 °C (10 h light/14 h dark) with watering for the cold stress experiment. The duration of the experiments was 4 weeks starting September 9, 2002. Control plants were maintained in the greenhouse and watered regularly. All plants were grown in 3-gallon pots in a mixture of 1 Perlite: 5.6 Metro Mix 510 (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA). Leaf and stem tissues were collected at weekly intervals from plants under drought, cold, and control conditions. Tissue samples were frozen in liquid nitrogen and stored at −80 °C until analyzed.

2.2. cDNA library construction, sequencing, and identification of dehydrin clones

Two cDNA libraries were constructed previously using RNA from cold acclimated and non-acclimated floral buds from the cultivar 'Bluecrop'. About 600 cDNA clones were picked at random from each library [24]. Nucleotide sequencing was initially performed from the 5′ ends of the cDNA inserts using Big Dye Terminator sequencing chemistry (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) by the University of Maryland, Center for Agricultural Biotechnology-DNA Sequencing Facility (College Park, MD, USA).

DNA sequences were trimmed of vector sequence manually or using the software package 'Lasergene' (DNASTAR Inc., Madison, WI, USA). Sequences were compared with the National Center for Biotechnology Information (NCBI) non-redundant protein database (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTX algo-

rithm [25] and default parameters. Individual ESTs were also assembled into contigs using 'Lasergene', with parameters optimized for ESTs rather than for genomic clones. Clones identified as dehydrins were examined individually and aligned using 'Lasergene' and/or Vector NTI™ (InforMax™, Frederick, MD, USA). Sequences of dehydrin clones were translated into all six possible reading frames using the Baylor College of Medicine Search Launcher: Sequence Utilities website (http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html) to identify the most likely open reading frame and putative translational start sites. A full-length clone identified as encoding the 14 kDa dehydrin, from peptide sequencing results (see below), was sequenced completely from both 5′ and 3′ ends by the DNA Sequencing Facility at Iowa State University (Ames, IA, USA).

2.3. Protein extraction, immunoblotting, and peptide sequencing

Protein was extracted from ∼500 mg frozen samples according to the method described by Lay-yee et al. [26]. Tissue samples were combined with 20 mg PVP-40 and ground with a mortar and pestle in liquid nitrogen. To this, 2.5 ml extraction buffer 1 (30 mM Tris–HCl pH 8.5 and 25 mM dithiothreitol) and 2.5 ml extraction buffer 2 (9% (w/v) SDS, 9% (w/v) sucrose, and 5% (v/v) β-mercaptoethanol) were added, thoroughly mixed, and transferred to polypropylene centrifuge tubes. The mixtures were heated for 10 min in a boiling water bath and spun at 16,300 × *g* for 60 min. The resultant supernatants were transferred to fresh tubes, three volumes of cold acetone were added and mixed, and the tubes placed at 4 °C overnight. Tubes were then spun at 4000 × *g* for 10 min to pellet total soluble proteins. The pellets were air-dried and resuspended in Laemmli [27] sample buffer. Protein was quantified visually using a modification of the Bradford dye binding assay [28] by spotting small volumes of the samples onto Whatman 3MM paper together with spots of a known concentration series of BSA, drying the paper thoroughly, and staining/destaining with Coomassie brilliant blue R-250.

Equal volumes of sample containing 30 μg protein were loaded on precast 10–20% SDS–polyacrylamide gels (Bio-Rad, Richmond, CA, USA). Gel electrophoresis was carried out in buffer containing 0.25 M Tris–HCl, 1.92 M glycine, and 1% (w/v) SDS using a Hoefer SE 600 standard vertical system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Separated proteins were electroblotted onto 0.2-μm nitrocellulose membranes (Schleicher and Schuell, Keane, NH, USA) using a semi-dry transfer cell (Bio-Rad) and transfer buffer (48 mM Tris–base, 39 mM glycine, 0.037% (w/v) SDS, and 20% (v/v) methanol) for 1.5 h at 0.8 mA/cm^2. Membranes were blocked at room temperature for 1 h with 5% (w/v) powdered milk in phosphate buffered saline or PBS (136 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, and 2 mM KH_2PO_4) and hybridized overnight at 4 °C with gentle rocking with a polyclonal antibody raised against a synthetic peptide of the consensus K box (EGGGL-MDKVKDKIHG) (Bio-Synthesis Incorporated, Lewisville, TX, USA) of blueberry dehydrins diluted 1:1500 in 5% (w/v) powdered milk in PBS. Blots were washed three times for 15 min each, at room temperature, with PBS + 0.02% (w/v) Tween 20 and incubated with secondary antibody at a 1:6000 dilution in 5% (w/v) powdered milk in PBS for 1 h. Finally, blots were washed with PBS + 0.02% (w/v) Tween 20 four times, 15 min each at room temperature. Immunoreactive bands were detected on X-ray film (Sterling Bioworld, Dublin, OH, USA) using a chemiluminescent immunoblot detection system with CDP-*Star* substrate (Western Star System, Tropix, Applied Biosystems, Bedford, MA, USA).

For peptide sequencing, 100 μg of soluble proteins from floral buds of 'Bluecrop', collected after 600 h of chilling to maximize dehydrin levels [11], were loaded in each of five lanes of a 12.5% SDS–PAGE gel. After gel electrophoresis, the gel was fixed in 12% (w/v) TCA for 1 h, stained with Coomassie brilliant blue R-250 in 25% methanol, and destained in 25% (v/v) methanol. The band believed to be the 14-kDa dehydrin was cut from the gel and shipped in 20% (v/v) methanol to the University of Florida, Protein Chemistry and Molecular Biomarkers Facility. The protein was subjected to a partial endoproteinase Lys-C digestion using one-half the amount of enzyme recommended in the protocol of Stone and Williams [29] for digestion of proteins in gels, and the resulting peptides were separated by reverse phase high-performance liquid chromatography (HPLC). Selected peptides were analyzed by mass spectrophotometry and sequenced in a gas-phase sequenator.

2.4. RNA extraction, preparation of Northern blots, and hybridizations

RNA was extracted from ∼600 mg frozen samples using the 'hot borate' protocol as described by Wilkins and Smart [30]. Total RNA (5 μg/lane) from each time point of the various treatments was separated on 1% agarose/formaldehyde gels, visualized and photographed to determine quality as well as confirm equal loading, and blotted onto BrightstarPlus™ nylon membranes (Ambion, Austin, TX, USA) by capillary transfer using the NorthernMax™ blotting and hybridization kit (Ambion). RNA was bound to the membranes by UV crosslinking.

For preparation of dehydrin probes, cDNA inserts were amplified from the plasmid clones using T7 forward and T3 reverse primers. The PCR products were precipitated with 1/4 volume 10 M ammonium acetate and 0.7 volumes propanol to remove the unincorporated nucleotides and then quantified by electrophoresing through 2% agarose gels with known concentrations of Low DNA Mass™ ladder (Invitrogen Life Technologies, Carlsbad, CA, USA). About 25 ng of each cDNA insert was ^{32}P-labeled to a specific activity of 1.0–2.0 × 10^8 cpm μg^{-1} by random priming

using the Megaprime™ DNA labeling system (Amersham Pharmacia Biotech). Membranes were prehybridized with ULTRAhyb buffer from the NorthernMax™ kit for 30 min and hybridized overnight at 42 °C. Blots were washed twice (15 min, 42 °C) with Low Stringency wash solution from the NorthernMax™ kit and exposed to X-ray film with intensifying screens at −80 °C.

3. Results and discussion

3.1. Isolation and sequence analysis of clones encoding the major 14 kDa dehydrin of blueberry

As part of a genomic project to identify genes associated with cold acclimation in blueberry, about 1200 cDNAs were previously sequenced, 600 each from two libraries constructed from RNA from non-acclimated and cold acclimated floral buds of the highbush cultivar 'Bluecrop' [24]. Of the ∼1200 cDNAs that were sequenced, 19 clones (10 from the cold acclimated library and nine from the non-acclimated library) were identified as encoding dehydrins by comparison to existing sequences in GenBank. These 19 dehydrin clones fell into three different clusters or contigs. The largest cluster, comprised of 15 clones, showed highest similarity with blueberry cDNA *bbdhn1*, encoding the major 60 kDa dehydrin ([16]; accession no. AAB84258). All clones within this cluster had inserts of ∼500–650 bp, which is an appropriate size to encode the major 14 kDa dehydrin of blueberry, and several appeared to be full length. The next largest cluster, comprised of three clones, was most similar to the dehydrin COR11 from *Poncirus trifoliata* ([31]; accession no. S59536]. All three clones within this cluster also had inserts in the ∼500–650 bp range and one appeared to be full length. The remaining singleton clone, with an insert of ∼1300 bp, was partial length and most similar to the blueberry dehydrin cDNA, *bbdhn5*, for which we had previously isolated a partial-length clone ([32]; accession no. AAF34606]. It was also very similar to *bbdhn1*. Homology to *bbdhn1* and *bbdhn5* was found throughout the coding sequence of this clone until near the carboxy terminus, after which there was a dramatic sequence divergence.

To determine if any of the clones possibly encode one of the major cold acclimation-associated dehydrins seen in blueberry floral buds, one clone representing each of the three contigs was used as a probe on Northern blots containing total RNA extracted from 'Bluecrop' floral buds collected at 0, 400, 800, and 1200 chill units (Fig. 1). Of the three, the clone, representing the contig with highest similarity to *bbdhn1* and inserts of ∼500–650 bp, hybridized to the most highly cold acclimation-responsive transcript (Fig. 1A). This ∼0.65 kb transcript was barely visible at 0 chill units and increased ∼45-fold (from densitometry) to maximum levels by 800 chill units. The length of the transcript was consistent with our conclusion that the largest

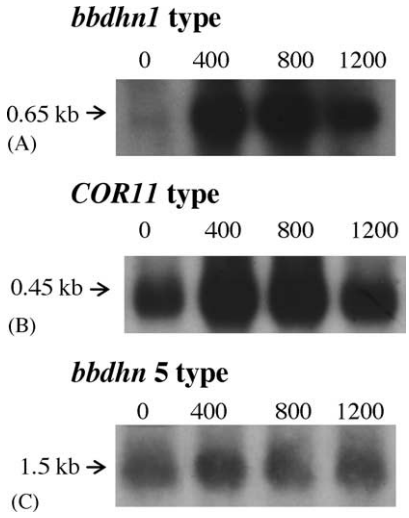

Fig. 1. RNA blot analysis of three different dehydrin contigs assembled from ESTs. One cDNA was selected from each contig (highest similarity to *bbdhn1* (A), *COR11* (B), and *bbdhn5*-type (C)) and used as probes on blots of total RNA extracted from 'Bluecrop' floral buds collected at 0, 400, 800, and 1200 chill units.

clones in this cluster may be full-length cDNAs. We previously showed that, under similar conditions, the *bbdhn1* cDNA hybridizes to two cold acclimation-responsive messages of ∼2.0 kb, the same size as the full-length *bbdhn1* cDNA, and ∼0.5 kb, the same size as the transcript seen here [16]. Consequently, we had speculated that the ∼0.5 kb transcript probably encodes the 14 kDa dehydrin. The current data strongly supports this hypothesis. The other clones representing contigs with highest similarity to COR11 and *bbdhn5* hybridized most strongly to messages of 0.45 and 1.5 kb, respectively (Fig. 1B and C). The 1.5 kb transcript did not appear to be induced with cold and would be too large to encode the 14 kDa dehydrin. The 0.45 kb transcript was present at a fairly high level before exposure to cold and its level increased only two- to three-fold to a maximum by 400 chill units.

An examination of the sequences of the clones comprising the putative 14 kDa dehydrin contig (highest similarity to *bbdhn1*) revealed the presence of two sequence subclasses that differ by only a few, single base, changes. Thus, since 'Bluecrop' is not a homozygous line, these two types probably represent two alleles of the same gene. All of the larger clones from this contig appeared to have the same predicted translational start and stop sites. The clone with the largest insert was completely sequenced from the 5′ and the 3′ ends. The complete nucleotide sequence, along with the deduced amino acid sequence, is shown in Fig. 2. The 653 bp sequence consists of a 55 bp 5′ UTR, an ORF of 303 bp, a 213 bp 3′ UTR, and an 82 bp polyA tail. Although there is no in-frame translational stop site in the 5′ UTR, the first Met is believed to be the start of translation since the first five amino acids (MAGIM) are identical to the translation start of the 60 kDa dehydrin and the ATG is surrounded by the consensus sequence (AaaATGGC)

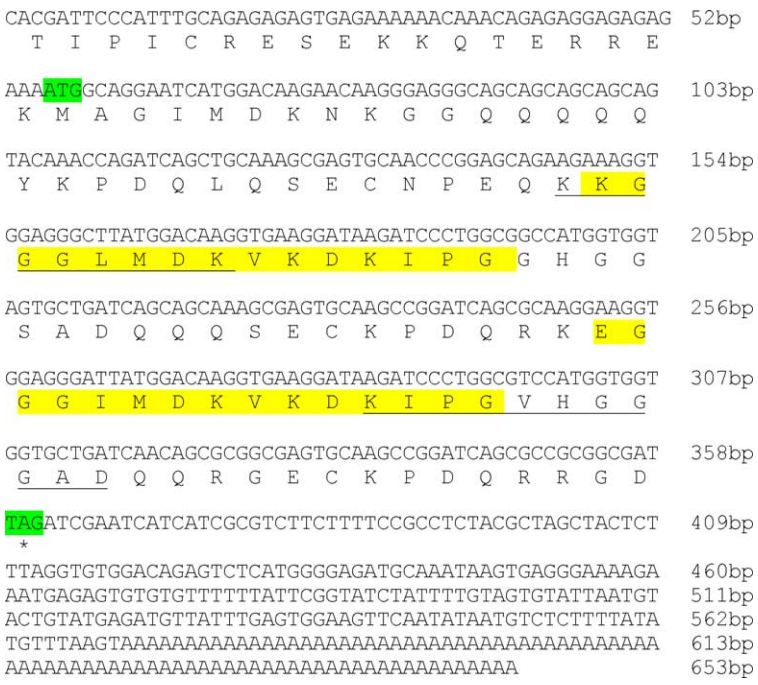

Fig. 2. Nucleotide and deduced amino acid sequence of the 653 bp dehydrin cDNA. The putative translational start and stop sites are highlighted. The deduced peptide sequences that match sequences obtained from the 14 kDa dehydrin are underlined.

common to plant initiation codons [33]. The clone has a deduced amino acid sequence of 101 residues, contains two lysine-rich K boxes indicative of dehydrins, and, like the 60 kDa dehydrin of blueberry, lacks the S and Y segments found in many dehydrins. Thus, this dehydrin can be classified as a K_2 type of dehydrin. Dehydrins without S and Y motifs (K_n types) are a rather rare class of dehydrins believed to be strongly induced by low temperature. Y_nSK_2 dehydrins are the most common type and are induced by dehydration; SK_n types are generally induced by both, dehydration and low temperature [1,21].

The predicted molecular mass of the protein encoded by the clone is 11 kDa, close to the size of the 14 kDa dehydrin. To confirm that the clone, whose sequence is shown in Fig. 2, encodes the 14 kDa major cold acclimation-associated dehydrin detected in blueberry floral buds, efforts were made to sequence the 14 kDa dehydrin, separated on an SDS–polyacrylamide gel. The N-terminus was blocked, so the protein was digested with endoproteinase Lys-C and two of the resulting peptides were sequenced (Table 1). Although all residues could not be determined with certainty, the peptide sequences that were obtained matched two sequences encoded by the clone (Table 1; Fig. 2), confirming that this clone does encode the 14 kDa dehydrin.

Table 1
Peptide sequences from the 14 kDa dehydrin and matching deduced amino acid sequences from the dehydrin cDNA clone

Peptide sequences from 14 kDa dehydrin	Matching deduced amino acid sequences from dehydrin cDNA clone
K[S/A/I/K]GGGLMDK	KKGGGLMDK
K[S/A/I/K]PGV[H/I][Y/G]GGAD	KIPGVHGGGAD

3.2. Expression of the 14 kDa dehydrin protein in response to cold and drought stress

Accumulation of the 14 kDa dehydrin was examined in time course experiments of cold-treated, drought-treated, and control plants using leaves and stems, because accumulation of the 14 kDa dehydrin has already been well documented in floral buds. Two cultivars, ‘Bluecrop’ and ‘Premier’, were chosen because they differ in their cold and drought tolerances with ‘Bluecrop’ being more cold and drought tolerant than ‘Premier’. Using a laboratory-controlled freeze–thaw regime, we have determined that the bud LT_{50}, lethal temperature that kills 50% of flower buds, for cold acclimated field plants of ‘Bluecrop’ is $-26\,^{\circ}C$ (ranges from -24 to $-29\,^{\circ}C$ in various years [11,23]) whereas that of ‘Premier’ is about $-22\,^{\circ}C$ (data unpublished). We previously found that ‘Bluecrop’ was also better able than ‘Premier’ to resist drought stress, probably by decreasing its consumption of water postponing dessication [18]. During the course of a 4–5 weeks drought treatment, the relative shoot water content of ‘Premier’ dropped to 51% of the control level while that of ‘Bluecrop’ dropped to only about 90% of the control level.

A polyclonal antibody previously raised against the gel-purified 65 kDa dehydrin cross reacts with the 65 and

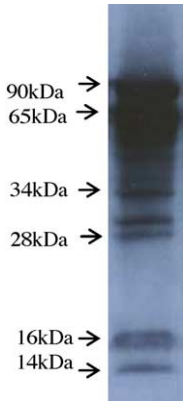

Fig. 3. Western blot of proteins from stems of cold stressed 'Premier' plants. Proteins were separated by SDS–PAGE followed by immunoblotting using polyclonal antiserum raised against a synthetic peptide of the consensus K box of blueberry dehydrins. The apparent molecular mass of selected cross-reacting proteins are shown to the left.

60 kDa dehydrins in blueberry but not the 14 kDa dehydrin [16]. This antibody was used earlier to study accumulation of the 65 and 60 kDa dehydrins in leaves, stems, and roots, in response to cold and drought stress but could not be used for studying expression of the 14 kDa dehydrin [18]. The 65 and 60 kDa dehydrins are glycosylated whereas the 14 kDa dehydrin is not [16]; therefore, a possible explanation for why this antibody does not cross react to the 14 kDa dehydrin is that it may react to glycosyl groups present on the 65 and 60 kDa dehydrins. To examine the accumulation of the 14 kDa dehydrin, a new polyclonal antibody was raised against a synthetic peptide of the consensus K box of all blueberry dehydrins sequenced to date (EGGGL-MDKVKDKIHG). This new antibody was found to cross react with the 65, 60, and 14 kDa dehydrins in addition to proteins of 16 and 90 kDa, and several in the 20–40 kDa range (Fig. 3). The preimmune serum control did not react to any proteins (data not shown).

3.2.1. Leaves

The 14 kDa dehydrin was not detected by immunoblot analysis in either 'Bluecrop' or 'Premier' leaves before drought treatment (Fig. 4A). However, a cross-reacting protein of 16 kDa was detected at the same level in leaves throughout the experiment. The 14 kDa dehydrin was just detectable in leaves of 'Premier' by 21 days of drought treatment and was barely visible in 'Bluecrop' by 28 days of drought stress. Level of the 14 kDa dehydrin remained about the same between 21 and 28 days of drought stress for 'Premier'. Neither the level of the 14 kDa dehydrin nor the 16 kDa dehydrin increased in control leaves over time, as judged by comparing the 0 day control with the 28 day control.

Levels of the 14 kDa dehydrin and 16 kDa cross-reacting protein increased significantly in leaves of both 'Bluecrop' and 'Premier' in response to cold treatment (Fig. 4B). We therefore consider the 16 kDa band to represent a new member of the blueberry dehydrin family. Levels of both low molecular weight dehydrins increased in 'Premier' and 'Bluecrop' by about 14 days of cold stress, and continued to rise throughout the course of the cold stress experiment. These results are consistent with our expectation that the 14 kDa dehydrin, a K_2-type of dehydrin, would be more strongly inducible by cold than by drought stress.

3.2.2. Stems

Immunoblots detected both the 14 and 16 kDa dehydrins in 0 day control stems of 'Bluecrop' while only the 14 kDa dehydrin was present in 0 day control stems of 'Premier' (Fig. 5A). Levels of both the 14 and 16 kDa dehydrins were elevated by 21 and 28 days of drought stress in stems of 'Bluecrop'. On the other hand, neither the 14 nor the 16 kDa dehydrin appeared to be induced with drought stress in 'Premier'. Rather, the 16 kDa dehydrin appeared to accumulate with time without drought stress, as judged by comparing the 0 and 28 day controls. These experiments

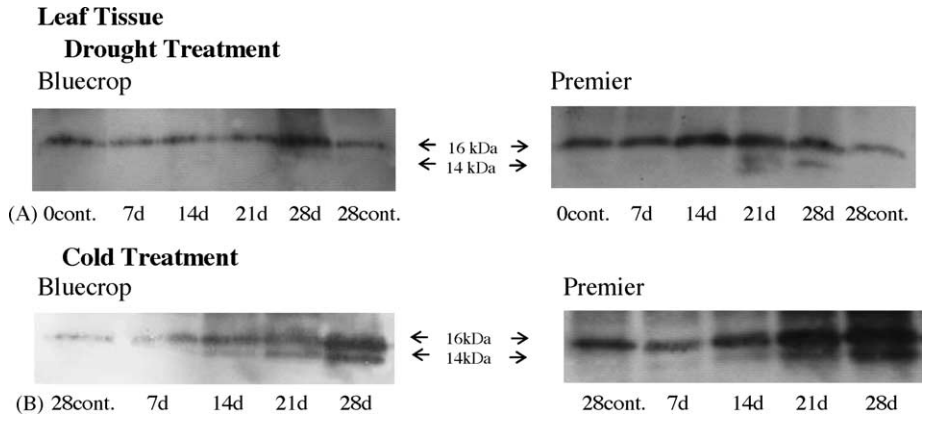

Fig. 4. Accumulation of the 14 kDa dehydrin protein in leaves of 'Bluecrop' and 'Premier' in response to drought (A) and cold stress (B). Proteins were extracted from leaves collected at weekly intervals (0, 7, 14, 21, and 28 days) of drought- (4 weeks without watering), cold- (4 °C for 4 weeks), and control- (well watered in a heated greenhouse for 4 weeks) treated plants. Equal amounts of proteins from 7-, 14-, 21-, and 28-day experimental plants and 0- and 28-day control plants (0cont. and 28cont.) were separated by SDS–PAGE and immunoblotted using polyclonal antiserum raised against a synthetic peptide of the consensus K box of blueberry dehydrins. The 14 kDa dehydrin and a previously uncharacterized 16 kDa dehydrin are indicated with arrows.

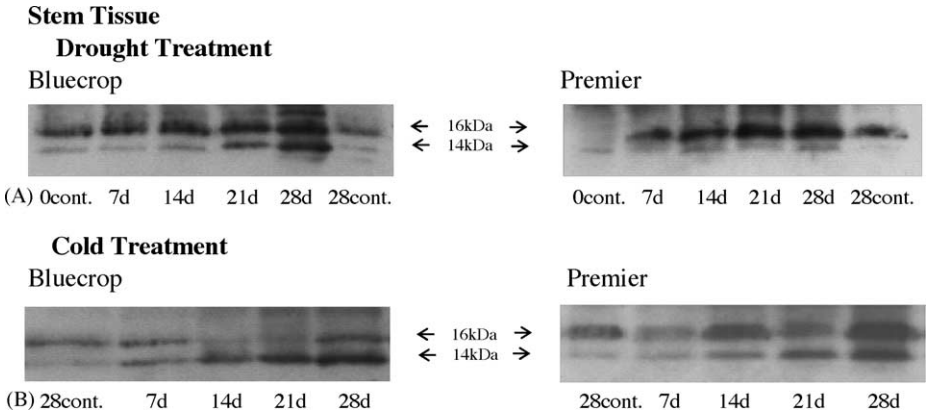

Fig. 5. Accumulation of 14 kDa dehydrin protein in stems of 'Bluecrop' and 'Premier' in response to drought (A) and cold stress (B). Proteins were extracted from stems collected at weekly intervals (0, 7, 14, 21, and 28 days) of drought- (4 weeks without watering), cold- (4 °C for 4 weeks), and control- (well watered in a heated greenhouse for 4 weeks) treated plants. Equal amounts of proteins from 7-, 14-, 21-, and 28-day experimental plants and 0- and 28-day control plants (0cont. and 28cont.) were separated by SDS–PAGE and immunoblotted using polyclonal antiserum raised against a synthetic peptide of the consensus K box of blueberry dehydrins. The 14 kDa dehydrin and a previously uncharacterized 16 kDa dehydrin are indicated with arrows.

were carried out starting September 9 and continued through October 7 as daylength gradually shortens. Our data suggests that the 16 kDa dehydrin may accumulate in stems in response to short photoperiods. There is a growing body of evidence from woody perennials that levels of some dehydrins do increase in response to short photoperiod [5,18,34,35] and that they are associated with the first stage of cold acclimation [19]. In 'Bluecrop', the 16 kDa dehydrin was already detectable at the start of the experiment, and did not accumulate further unless the plants were drought stressed. This is consistent with 'Bluecrop', a northern highbush variety, having a longer critical photoperiod (longest photoperiod that elicits a short-day response [36]) than 'Premier', a southern rabbiteye variety.

Level of the 14 kDa dehydrin increased steadily in stems of 'Bluecrop' and 'Premier' in response to cold treatment and did not appear to be affected with time in the controls (Fig. 5B). Level of the 16 kDa dehydrin, on the other hand, did not increase in response to cold stress in either 'Bluecrop' or 'Premier' but, as stated above, increased in 'Premier' stems over time (by comparing 0 and 28 day controls from drought experiment).

3.3. Accumulation of 14 kDa dehydrin mRNA in response to cold and drought stress

To determine if changes in levels of the 14 kDa dehydrin protein are a reflection of changes in transcript levels, a PCR

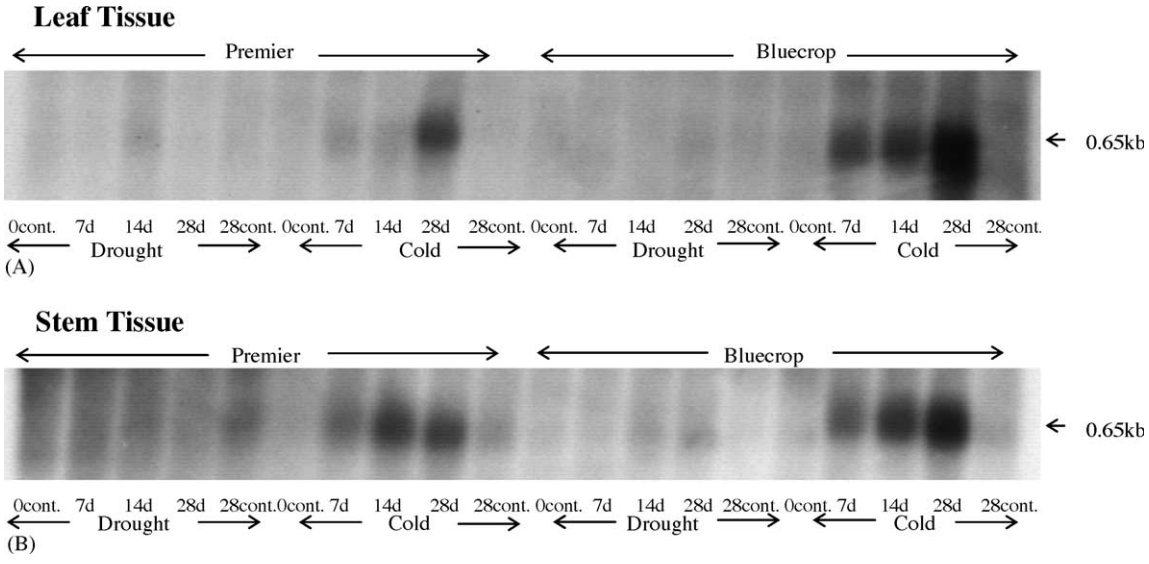

Fig. 6. Accumulation of 14 kDa dehydrin mRNA in leaves (A) and stems (B) of drought- and cold-treated 'Premier' and 'Bluecrop' plants. RNA was extracted from leaves and stems collected from 0- and 28-day control plants, and from 7-, 14-, and 28-day drought- and cold-stressed experimental plants. Equal amounts of RNA (5 μg) from the various samples were separated on formaldehyde gels and hybridized with the 14 kDa dehydrin cDNA. The hybridizing 0.65 kb fragments are indicated.

fragment of the entire 14 kDa dehydrin cDNA clone was used as a probe on blots of RNA from cold- and drought-treated 'Bluecrop' and 'Premier' plants. The 14 kDa dehydrin cDNA probe hybridized strongly to a single transcript of about 650 bases, the same size as the full-length cDNA clone (Fig. 6). When the blots were overexposed, cross-hybridization to a 2.0 kb message, corresponding to the 60 kDa dehydrin transcript, could be detected as well (data not shown).

3.3.1. Leaves

The levels of 14 kDa dehydrin mRNA correlated well with protein levels detected in both 'Bluecrop' and 'Premier' under either drought or cold stress. The 14 kDa dehydrin mRNA level did not appear to change over time nor was it elevated significantly in response to drought treatment in leaves of either 'Bluecrop' or 'Premier' (Fig. 6A). The level increased in response to cold stress as early as 7 days of treatment, and continued to increase throughout the 28 days of treatment in leaves of both genotypes. However, the levels of the 14 kDa dehydrin mRNA were always higher in leaves of cold-treated 'Bluecrop' plants than in 'Premier' plants.

3.3.2. Stems

The 14 kDa dehydrin mRNA increased slightly in abundance by 14 days of drought stress and remained elevated at 28 days in 'Bluecrop' stems (Fig. 6B). In 'Premier', the amount of 14 kDa dehydrin mRNA did not increase in response to drought stress, nor was it elevated significantly in either genotype over time. Cold stress resulted in an increase in the 14 kDa dehydrin mRNA level within 7 days in both genotypes, but the increase was greater in 'Bluecrop' than 'Premier' stems by 28 days. Again, these results were consistent with expression patterns of the 14 kDa dehydrin at the protein level.

4. Conclusions

Here, we report obtaining and sequencing a full-length cDNA clone encoding the major cold acclimation-associated 14 kDa dehydrin characterized previously in floral buds of blueberry [11,13]. The sequence of this clone indicated that it is a K_2-type dehydrin with high similarity to the previously isolated full-length cDNA clone encoding the major cold acclimation-associated 60 kDa dehydrin, K_5-type, of blueberry. Expression of the 14 kDa dehydrin gene was examined at the protein and RNA levels in time course experiments using leaves and stems of cold-treated, drought-treated, and control plants of two blueberry cultivars, 'Bluecrop' and 'Premier', which differ in terms of their cold and drought tolerances. Expression at the protein level was monitored on Western blots using a polyclonal antibody raised against a synthetic peptide of the consensus K box of blueberry dehydrins. Expression at the RNA level was monitored on Northern blots using a full-length 14 kDa dehydrin PCR fragment as probe. The 14 kDa dehydrin protein appeared to be induced primarily with cold stress and not at all, or to a lesser extent in some cases, with drought stress, and was induced in all the blueberry organs examined, which was similar to our earlier findings with the 65 and 60 kDa dehydrins of blueberry [18]. For example, the level of the 14 kDa dehydrin was elevated significantly in leaves and stems of both genotypes in response to cold stress but just slightly in leaves of 'Bluecrop' and 'Premier' and only in stems of 'Bluecrop' in response to drought stress. This is similar to findings on K_n type dehydrins in other plants, too, such as the wheat *Wdhn13* gene, whose expression is predominantly responsive to low temperature treatment, although it also responds to dehydration stress to a lesser extent [21]. A previously uncharacterized 16 kDa dehydrin was detected with the new antibody and appeared to increase in abundance over time during the course of the experiments in stems of the southern variety 'Premier', perhaps in response to short photoperiods. At the time of these experiments (September through October), the critical photoperiod for the northern variety 'Bluecrop' may have already passed. Also, level of the 16 kDa dehydrin appeared to increase in leaves, but not stems, of both genotypes in response to cold stress and in stems of 'Bluecrop' with drought stress. Patterns of expression of the 14 kDa dehydrin at the protein level were very consistent with patterns at the RNA level, and the 14 kDa dehydrin message accumulated to higher levels in the more cold hardy and drought tolerant genotype, 'Bluecrop', than in 'Premier'.

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